Biochimica et Biophysica Acta 1808 (2011) 717–726

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Non-reducing trisaccharide fatty acid monoesters: Novel detergents in membrane biochemistry

Ignacio Pérez-Victoria [a,*,1], Francisco J. Pérez-Victoria [a], Sándalo Roldán-Vargas [b], Raquel García-Hernández [a], Luis Carvalho [a], Santiago Castanys [a], Francisco Gamarro [a], Juan C. Morales [c,*], José M. Pérez-Victoria [a,*]

[a] Instituto de Parasitología y Biomedicina "López-Neyra", CSIC, Parque Tecnológico de Ciencias de la Salud, Avda. del Conocimiento s/n, 18100 Armilla, Granada, Spain
[b] Grupo de Física de Fluidos y Biocoloides, Departamento de Física Aplicada, Universidad de Granada, 18071 Granada, Spain
[c] Department of Bioorganic Chemistry, Instituto de Investigaciones Químicas, CSIC-Universidad de Sevilla, 49 Américo Vespucio, 41092 Sevilla, Spain

ARTICLE INFO

Article history:
Received 27 July 2010
Received in revised form 18 November 2010
Accepted 22 November 2010
Available online 3 December 2010

Keywords:
Carbohydrate fatty acid monoesters
Detergents
Membrane protein purification
(Proteo)liposomes
ABC transporters

ABSTRACT

Three families of non-reducing trisaccharide fatty acid monoesters bearing C_{10} to C_{18} acyl chains have been prepared by enzymatic synthesis in organic media. Their critical micelle concentrations, determined by dye-inclusion measurements, cover a broad range from mM to μM. The new compounds are capable of dissolving phospolipid vesicles and have been characterized as detergents in membrane biochemistry. In a comparative screening test for solubilizing/extraction capacity under native conditions of an ABC transporter as model integral membrane protein, the novel detergents have shown an excellent behavior similar to other commercial carbohydrate-based detergents and in some cases even better than the commonly employed β-dodecylmaltoside. The new detergents are also efficient at extracting membrane proteins from different lipidic environments and are likewise compatible with common protein affinity chromatography purification. These compounds may also be used for the preparation of (proteo)liposomes by detergent removal, not only using the classical method of detergent adsorption on hydrophobic resins but also by enzyme-catalyzed hydrolysis of the ester bond. These results show the new detergents as promising tools to expand the arsenal for membrane protein studies.

1. Introduction

Sugar-based surfactants are an important type of non-ionic amphiphiles constituted of carbohydrates as polar head groups conjugated with long-chain alcohols, fatty acids, or other hydrophobic molecules; these compounds have broad applications in food, cosmetic, and pharmaceutical industries [1]. Two of the most important classes of carbohydrate-based surfactants are alkyl glycosides and sugar fatty acid esters [2]. Carbohydrate fatty acid esters are produced industrially by acid-catalyzed esterification or transesterifications [3]. Monoesters, which have better solubility in water than higher substituted derivatives, are comprised of a mixture of regioisomers due to the nature of the chemical process [4,5]. Nevertheless, enzymatic catalysis allows in some cases the regioselective esterification of carbohydrates [6]. The effect of the sugar, the fatty acid chain length, and even the acylation position on the surfactant and self-organizing properties of carbohydrate fatty acid monoesters have also been studied [7–10].

Sugar-based surfactants have been applied as detergents in membrane protein extraction, purification, and crystallization [11,12]. Structure–function relationship studies of integral membrane proteins require the use of mild nondenaturing detergents capable of solubilizing and stabilizing the protein of interest by maintaining its biologically active native folding [13]. Carbohydrate-based detergents meet these requirements and have been successfully employed to solubilize, purify, and reconstitute membrane proteins. Probably the most popular detergents derived from sugars used in biochemistry are alkyl glycosides, such as octylglucoside (OG) and dodecylmaltoside (DDM), which are available as pure anomers [14]. Sucrose fatty acid monoesters are also commercially available and likewise have been employed in

Abbreviations: ABC, ATP-binding cassette; MDR, multidrug resistance; Pgp, P-glycoprotein; Ni-NTA, nickel nitriloacetic acid; HLB, hydrophilic–lipophilic balance; CMC, critical micelle concentration; DDM, β-dodecylmaltoside; OG, β-octylglucoside; SUL, sucrose monolaurate; R6D, 6-*O*-decanoylraffinose; R6L, 6-*O*-lauroylraffinose; R6M, 6-*O*-myristoylraffinose; R6P, 6-*O*-palmytoylraffinose; R6S, 6-*O*-stearoylraffinose; R1D, 1″-*O*-decanoylraffinose; R1L, 1″-*O*-lauroylraffinose; R1M, 1″-*O*-myristoylraffinose; R1P, 1″-*O*-palmytoylraffinose; R1S, 1″-*O*-stearoylraffinose; MD, decanoylmelezitose; ML, lauroylmelezitose; MM, myristoylmelezitose; MP, palmitoylmelezitose; MS, stearoylmelezitose

* Corresponding authors. J.M. Pérez-Victoria is to be contacted at tel.: +34 958181658x667; fax: +34 958181632. J.C. Morales, tel.: +34 954489561; fax: +34 954460565. I. Pérez-Victoria, tel.: +34 958993965x7061; fax: +34 958846710.
E-mail addresses: ignacio.perez-victoria@medinaandalucia.es (I. Pérez-Victoria), jcmorales@iiq.csic.es (J.C. Morales), josepv@ipb.csic.es (J.M. Pérez-Victoria).
[1] Present address: Fundación MEDINA, Centro de Excelencia en Investigación de Medicamentos Innovadores en Andalucía, Parque Tecnológico de Ciencias de la Salud, Avda. del Conocimiento s/n, Edif. Centro de Desarrollo Farmaceútico, 18100 Armilla, Granada, Spain.

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.11.031

membrane protein research [15], although in this case, the commercial sucrose monoesters are comprised of a mixture of regioisomers [5].

To ensure reproducible membrane protein extraction, the use of homogeneous and well-defined surfactants is of the utmost importance and this requirement is still more critical in membrane protein crystallization experiments [16,17]. New sugar derivatives with improved performance are desired to augment the range of currently available detergents. In this context, Brumer and co-workers have recently reported the synthesis of a novel family of surfactants derived from highly branched xyloglucan oligosaccharides and their usefulness in extracting membrane-bound enzymes of plant origin [18].

We have decided to extend our recently described regioselective enzymatic oligosaccharide acylation methodology [19,20] to prepare new homologous series of non-reducing trisaccharide fatty acid monoesters, i.e., using fatty acids of different chain length, in order to study their properties and application in membrane biochemistry. The use of trisaccharides as polar head groups allows access to long-chain derivatives, which display much higher solubility in water at room temperature than, for example, monopalmitoyl and monostearoyl esters of sucrose [21]. Herein, we describe the enzymatic synthesis of three novel families of fatty acid monoesters derived from raffinose (**1**) and melezitose (**2**) (Fig. 1), the characterization of their critical micelle concentration (CMC), and their application as detergents in biochemistry both for membrane protein extraction and purification and for (proteo)liposome preparation. As a model integral membrane protein, we have used BmrA [22], an ABC (ATP-binding cassette) transporter from *Bacillus subtilis*. Interestingly, His-tagged BmrA can be highly overexpressed in the plasma membrane of *Escherichia coli* [23] and present the highest ATPase activity reported so far for an ABC transporter [22]. In addition, its solubilization with DDM, purification, and reconstitution into proteoliposomes or in 2D crystals have already been described [22,24]. These characterizations facilitate the monitoring of its solubilization in a native form by the newly synthesized detergents and their comparison with the effect of DDM.

2. Materials and methods

2.1. Chemicals

Anhydrous pyridine, vinyl decanoate, vinyl laurate, *n*-dodecyl-β-D-maltoside and sucrose monolaurate were supplied by Fluka; vinyl myristate and vinyl palmitate from TCI; vinyl stearate, anhydrous tert-butanol, methanol-d_4, molecular sieves (3 Å, 8–12 mesh), and methyl-β-cyclodextrin (MβCD) from Aldrich; ATP, Bradford reagent, protease inhibitor cocktail, phosphatidylcholine, raffinose, melezitose, SDS, and CHAPS from Sigma. Granulated lipase from *T. lanuginosus* (Lipozyme TL IM), liquid lipase preparation from *T. lanuginosus* (Lipozyme TL 100 L), and subtilisin Carlsberg (purified powder) were kindly donated by Novozymes A/S. Molecular sieves were preactivated at ca. 350 °C for 12 h. Reactions were monitored by TLC on precoated Silica-Gel 60 plates (Alugram Sil G/UV254 supplied by Macherey-Nagel) and detected by heating with Mostain (500 ml of 10% H_2SO_4, 25 g of $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$, 1 g of $Ce(SO_4)_2\cdot 4H_2O$) or 10% H_2SO_4 in ethanol. The elution system was $CHCl_3$–MeOH (2.5/1). Monoesters were purified by flash chromatography with Aldrich Silica gel 60 (200–400 mesh) using a gradient of chloroform/methanol 5:1 to 2:1 (v/v). HPLC gradient grade acetonitrile was purchased from Sharlab. Water was purified with a Milli-Q plus system (Millipore, Bedford MA, USA). For the preliminary isomerization study followed by LC-ESI-MS (see Supplementary data), three regioisomers were prepared as described previously: 6-*O*-lauroylraffinose and 1″-*O*-lauroylraffinose [19] and 2′-*O*-lauroylraffinose [25].

2.2. Analytical procedures

LC-ESI-MS monitorization of the isomerization of regioisomeric raffinose monolaurates (see Supplementary data) was carried out following a previously optimized protocol using a Waters Alliance 2695 separation module coupled to a Waters Micromass ZQ single quadrupole mass spectrometer [5]. Regioisomeric purity in the corresponding isolated monoesters of each homologous series was determined by ^{1}H NMR analysis and also observed qualitatively by positive-ion ESI mass spectrometry (direct infusion) at high cone voltage to induce in-source fragmentation and comparing the relative intensities of the main obtained fragments as previously described [26]. NMR spectra were recorded on either a Bruker AVANCE 300 or ARX 400 [300 or 400 MHz (^{1}H) and 75 or 100 MHz (^{13}C)] at room temperature for solutions in CD_3OD. Chemical shifts are referred to the methanol multiplet, centered at 3.31 ppm for ^{1}H NMR and 49.0 ppm for ^{13}C NMR. The ^{1}H NMR spectra of the corresponding decanoyl, myristoyl, palmitoyl, and stearoyl monoesters in each homologous series showed chemical shifts and coupling constants within ±0.005 ppm and ±0.5 Hz, respectively, of those found for the lauroyl derivatives [19]. Likewise, resonances for the trisaccharide carbon atoms in the ^{13}C NMR spectra of the corresponding decanoyl, myristoyl, palmitoyl, and stearoyl monoesters in each homologous series showed chemical shifts within ±0.1 ppm of those found for the lauroyl derivatives [19]. Optical rotations for pure regioisomers (those trisaccharide monoesters obtained with >95% regioselectivity) were measured (Sodium D line) at 20 °C with a Perkin-Elmer 241 polarimeter. High-resolution FAB (+) mass spectral analyses were obtained on a Micromass AutoSpec-Q spectrometer. Infrared spectra were recorded using a Nicolet 20SXB FTIR spectrophotometer.

2.3. Enzymatic synthesis of the homologous series of trisaccharide fatty acid monoesters

Enzymatic acylations were carried out essentially as previously described [19] using for each desired aliphatic chain length the corresponding vinyl fatty acid ester (decanoate, laurate, myristate, palmitate, and stearate) as activated acylating agent and the carbohydrates in their amorphous form prepared by lyophilization of the corresponding aqueous solutions.

The homologous series 6-*O*-acylraffinose and monoacylmelezitose were prepared in the following way using the lipase Lipozyme TL IM as biocatalyst: the carbohydrate (504 mg, 1 mmol) was dissolved in anhydrous pyridine (12 ml) at 60 °C before careful addition of preheated (60 °C) anhydrous *tert*-butanol (15 ml); then Lipozyme TL IM (250 mg) and 3 Å molecular sieves (250 mg) were added and the suspension was maintained 30 min at 60 °C with orbital shaking (250 rpm); finally, the corresponding vinyl fatty acid ester (3 mmol) was added. When conversion stopped as judged by TLC (around 48 h), the mixture was cooled and filtered. The solvent was evaporated under vacuum at 45 °C eliminating last traces of pyridine by co-evaporation with toluene. The remaining residue was subjected to flash chromatography. Concentration of pure fractions *in vacuo* afforded the corresponding trisaccharide fatty acid monoesters as amorphous white solids. Compounds spectroscopic characterizations are available as Supplementary data.

The homologous series 1″-*O*-acylraffinose was prepared in the following way using a MβCD–subtilisin Carlsberg preparation as biocatalyst [19]. In brief, raffinose (504 mg, 1 mmol) was dissolved in anhydrous pyridine (8 ml) at 40 °C before addition of the corresponding vinyl fatty acid ester (3 mmol). The mixture was shaken (250 rpm) in the presence of MβCD–subtilisin Carlsberg (180 mg). When conversion stopped as judged by TLC (around 48 h), the mixture was cooled and filtered. The solvent was evaporated under vacuum at 45 °C eliminating last traces of pyridine by co-evaporation with toluene. The remaining residue was subjected to flash chromatography. Concentration of pure fractions *in vacuo* afforded the corresponding raffinose fatty acid monoesters as amorphous white solids. Compounds spectroscopic characterizations are available as Supplementary data.

2.4. Colorimetric determination of the CMC

Estimation of the CMC for the compounds was carried out by a dye-inclusion assay previously developed for non-ionic surfactants [27]. Instead of preparing a solution of the dye (Coomassie brilliant blue G-250) as described in the original paper, a commercial solution of Bradford reagent was employed. The method was adapted to microscale in the following manner: in a microfuge tube, 75 μl of each surfactant solution in Milli-Q® water and 225 μl of Bradford reagent were mixed thoroughly by vortexing a few seconds; 175 μl of each mixture was transferred to the wells of a 96-well microtiter plate and the absorbance at 620 nm was measured in a plate reader (Bio-Rad model 550). Absorbance was plotted against surfactant concentration (logaritmic scale), and the CMC was taken as the breakpoint of each curve [18]. For the reference detergents (except the anionic SDS), the CMC was determined in the same manner rendering the following values: CHAPS (1.8 g/l; 2.3 mM), DDM (0.06 g/l; 116 μM), SUL (0.085 g/l; 162 μM), which correlate well with reported ones [28].

2.5. Vesicle solubilization test

Unilamellar phosphatidylcholine (PC) liposomes of 100 nm mean diameter were prepared as previously described [29] and diluted to a final concentration of 1 mg lipid/ml. The solubilization of these vesicles with 6-*O*-lauroylraffinose (R6L) was followed by measuring the changes in turbidity (OD at 400 nm measured in a Varian Cary spectrophotometer) as a function of detergent concentration as previously described for octylglucoside and Triton X-100 [30].

2.6. Screening of the new detergents for solubilizing/extraction capacity of the protein BmrA under native conditions

E. coli BmrA-enriched membranes were prepared as described [23] and kept frozen (as aliquots) in liquid nitrogen. Protein concentration of membranes was estimated by a bicinchoninic acid (BCA) assay (Pierce) after precipitation of proteins by trichloroacetic acid. Solubilization of the membranes with the detergents (homologous series of raffinose and melezitose monoesters plus controls) was carried out as follows (150 μl total volume): membranes were rapidly defrosted at 37 °C and diluted into the solubilization buffer containing 50 mM HEPES/NaOH (pH 8), 15% glycerol, 100 mM NaCl, 10 mM imidazole, 1 mM DTT, protease inhibitors mixture (10 μl/ml buffer), and the corresponding detergent (1% or 10× CMC) to a final protein concentration of 1.5 mg/ml. The suspension was stirred for 1-h incubation at 4 °C (except for the SDS control, which was carried out at room temperature), and the insoluble material was removed by centrifugation (150,000 *g*, 1 h, 4 °C). Supernatants were analyzed by SDS-PAGE (Coomassie brilliant blue stained) and the total protein concentration in them was also estimated by the BCA assay. ATPase-specific activity in the supernatants was measured by Pi release based on a colorimetric method as previously described [31], using 50 mM HEPES/NaOH (pH 8), 150 mM NaCl, 5 mM $MgCl_2$, 2 mM NaN_3, 2 mM ATP, and a 20-min assay time at 37 °C in the absence and in the presence of vanadate (250 μM). Detergent concentration was kept at the CMC. Activity is expressed in nmol of Pi liberated/min per mg of total protein, and for comparison purposes, the activity observed in the DDM supernatant was assigned an arbitrary 100%.

2.7. Solubilization of different membrane proteins by 6-O-lauroylraffinose (R6L)

Membranes from wild-type or MDR *L. tropica* lines or from MDR CH^RB30 mammalian cells were obtained as described [32,33]. Membrane proteins were solubilized with R6L as described above and the proteins of interest were identified by Western blot using the primary antibodies indicated.

2.8. Purification of BmrA

6-*O*-lauroylraffinose (R6L) was the selected detergent for the protein purification using the methodology previously described for BmrA purification using DDM as detergent. BmrA-enriched membranes were solubilized using the previous procedure with 1% detergent and scaling-up to a final solubilization volume of 7 ml. All the following purification steps were carried out at 4 °C. Solubilized membrane proteins were incubated for 45 min with Ni-NTA agarose (1 ml of resin), which was previously equilibrated in the solubilization buffer. After incubation, the resin was transferred to a column (1×5 cm) and washed with 60 ml of washing buffer (50 mM HEPES/NaOH pH 8, 15% glycerol, 100 mM NaCl, 20 mM imidazole, 5 mM β-mercaptoethanol, and 0.05% R6L). The protein was recovered with elution buffer (50 mM HEPES/NaOH pH 8, 15% glycerol, 50 mM NaCl, 250 mM imidazole, 5 mM β-mercaptoethanol, and 0.05% R6L). The fractions (500 μl each) were analyzed for protein content and purity by SDS-PAGE. Pure fractions of similar concentration were pooled, and imidazole was removed with a desalting column using as equilibration and elution buffer 50 mM HEPES/NaOH pH 8, 15% glycerol, 50 mM NaCl, 5 mM β-mercaptoethanol, and 0.05% R6L. Purified BmrA was frozen and stored in liquid nitrogen. ATPase activity was measured for the purified protein in the absence and the presence of lipids (25 mg lipid/mg protein) as described before (50 mM HEPES/NaOH pH 8, 150 mM NaCl, 10 mM $MgCl_2$, 4 mM NaN_3, 10 mM ATP) for the supernatants but using a regeneration ATP system (8 mM phosphoenolpyruvate and 120 μg/ml pyruvate kinase) [31,34].

2.9. Preparation of (proteo)liposomes

Preparation of liposomes by detergent removal from mixed micelles of detergent (R6L or DDM) and lipids (*E. coli* total lipid extract—Avanti Polar Lipids) employing Bio-Beads SM2 (Bio-Rad) was carried out as previously described [34].

For the preliminary test of enzymatic hydrolysis of the trisaccharide monolaurate esters, a covalently immobilized form of *T. lanuginosus* lipase onto Eupergit C was prepared as previously described [35]. The biocatalyzed hydrolysis of 6-*O*-lauroylraffinose (R6L), 1″-*O*-lauroylraffinose (R1L), and melezitose monolaurate (ML) was carried out similarly to the reported lipase-catalyzed hydrolysis of 6-*O*-octanoylglucose [36]: basically 50 mg of the immobilized lipase was added to 500 μl of 50 mM HEPES/NaOH (pH 8) containing 5 mg of the corresponding trisaccharide monolaurate, the reaction mixture was incubated at room temperature with orbital stirring and hydrolysis was monitored over time by TLC, a control sample with no enzyme (just Eupergit C) was included.

Proteoliposomes containing the purified BmrA (with R6L as detergent) were reconstituted by adding 50 μg of purified protein to mixed micelles containing *E. coli* lipids total extract (2 mg/ml) and R6L (0.2%) in a final volume of 500 μl (50 mM HEPES/NaOH pH 8) and removing the detergent using Bio-Beads (20 mg×3 additions) as described for the case of DDM as detergent [34]. Reconstitution by enzymatic hydrolysis using the immobilized *T. lanuginosus* lipase preparation was performed in the same way but using 10 mg of biocatalyst instead of the Bio-Beads. ATPase activity was measured for the reconstituted liposomes as described before for the purified protein.

2.10. Liposome characterization

Liposome sizes were determined by means of dynamic light scattering (DLS) using a slightly modified Malvern 4700 System (UK) working with an He–Ne laser beam (wavelength *in vacuo* 632.8 nm). The scattered intensity autocorrelation functions were measured and recorded at different scattering angles within the range [60–120°]. Data analysis was performed using our own computer software,

assuming free Brownian motion [37]. In this respect, suspensions were sufficiently diluted to avoid undesirable long-range interactions. Accordingly, the final liposome diameters were obtained as the average of 20 independent DLS measurements, whereas their associated absolute errors correspond to the standard deviation of these measurements. Lamellarity of the formed liposomes was determined by a (palmitoyl, C_6-NBD)-PC (Avanti Polar Lipids) fluorescence dithionite reduction assay as previously described [38]. Fluorescence measurements were performed at 25.0 ± 0.1 °C using a SLM-Aminco 8000C spectrofluorometer. Liposomes were also characterized by negative staining of the suspensions with 2% uranile acetate and further visualization by transmission electron microscopy (TEM).

3. Results and discussion

3.1. Enzymatic synthesis of homologous series of raffinose and melezitose fatty acid monoesters

We have prepared families of raffinose and melezitose fatty acid monoesters carrying the acyl group just on primary hydroxyls (Fig. 1) to avoid intramolecular transacylation in aqueous solutions. In fact, a preliminary study by LC-ESI-MS to examine possible isomerization in three regioisomers, 6-*O*-lauroylraffinose and 1″-*O*-lauroylraffinose [19], acylated on primary hydroxyls, and 2′-*O*-lauroylraffinose [25], acylated on a secondary hydroxyl, showed that 2′-*O*-lauroylraffinose isomerizes quickly, while negligible transacylation is observed for the other two regioisomers acylated on primary hydroxyls; also residual hydrolysis of the ester was observed in all cases after a couple of days (see Fig. S1 in Supplementary data).

In order to cover a wide range of hydrophilic–lipophilic balance (HLB) [39,40] and CMC values, we decided to prepare decanoyl, lauroyl, myristoyl, palmitoyl, and stearoyl monoesters of raffinose and melezitose. Based on our previously reported results [19], two enzymes that regioselectively acylate primary hydroxyls in these non-reducing trisaccharides were chosen: silica-granulated lipase from *Thermomyces lanuginosus* (Lipozyme TL IM) and the alkaline protease from *Bacillus licheniformis*, subtilisin Carlsberg colyophilized with methyl-β-cyclodextrin. The vinyl esters of the corresponding fatty acid were employed as activated acylating agents, and we extrapolated the reaction conditions already optimized to prepare monolauroyl esters of these trisaccharides [19].

Yields of isolated monoester are summarized in Table 1. It can be observed that these are independent of the chain length of the fatty acid when the reaction is catalyzed by the lipase, in accordance with previously reported values by Ballesteros and co-workers for the synthesis of malotriose monoesters using a celite immobilized preparation of the same lipase [41]. On the other hand, for the reactions catalyzed by the protease, an almost linear decay in yield is observed when the fatty-acid chain length is increased. A similar trend in catalytic activity has also been observed for the enzymatic synthesis of sucrose fatty acid monoesters with the analogous protease from *B. subtilis* [10,42].

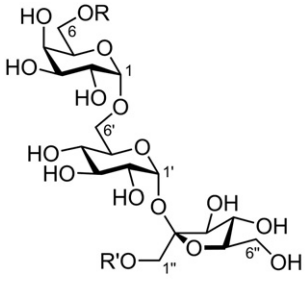
Table 1
Yields of isolated monoester in the different enzyme-catalyzed transesterifications of the trisaccharides using the indicated activated acylating agents.

Acylating agent	Subtilisin Carlsberg–MβCD	Lipozyme TL IM	
	Raffinose	Raffinose	Melezitose
Vinyl decanoate	74%	80%	56%
Vinyl laurate	57%	79%	52%
Vinyl myristate	44%	79%	51%
Vinyl palmitate	25%	81%	55%
Vinyl stearate	13%	84%	53%

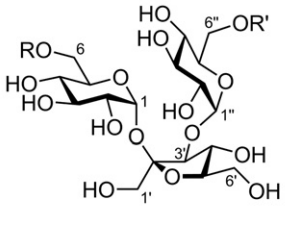
The regioselectivity (see Supplementary data) was independent of the acyl chain length in the reactions catalyzed by the lipase. With raffinose as substrate, only the 6-*O*-acyl derivatives were obtained (>99% regioisomeric purity). For melezitose, a mixture ca. 2:1 of the 6-*O*-acyl/6″-*O*-acyl derivatives was obtained with all the fatty acids. In the case of the reactions catalyzed by subtilisin Carlsberg–MβCD, the regioselectivity is affected by the alkyl chain length: with vinyl decanoate as acylating agent, almost only 1″-*O*-decanoylraffinose (97% regioisomeric purity) is produced while when using the longest vinyl stearate a mixture of 1″-*O*-stearoylraffinose/6-*O*-stearoylraffinose in a ratio 82:18% was obtained. A similar decrease in the regioselectivity of the acylation when increasing the length of the alkyl chain has also been described for the synthesis of sucrose fatty acid monoesters catalyzed by the protease from *B. subtilis* [42]. Computational studies about the acylation of sucrose catalyzed by subtilisin Carlsberg suggest that when the fatty acid chain length increases, acylation in position 1′ becomes more sterically hindered while acylation in position 6 becomes less sterically hindered [43]. It could be possible to apply the same reasoning to raffinose since this trisaccharide can be considered as a 6-*O*-α-galactopyranosylsucrose.

Thus, we have prepared three families of non-reducing trisaccharide fatty acid monoesters (Fig. 1): the first one is a homologous series of 6-*O*-acylraffinose (**1a**) regioisomerically pure (>99%); the second one is a homologous series of raffinose monoesters comprised for each alkyl chain mainly by 1″-*O*-acylraffinose (**1b**); the third one is a homologous series of melezitose monoesters comprised for each alkyl chain length of a mixture ca. 2:1 of the regioisomers acylated in positions 6/6″ (**2a**/**2b**). HLB values for these compounds range ca. 13–15 (Table 2) and, according to Griffin's classification [39,44], are adequate surfactants to prepare oil-in-water emulsions and could be employed as detergents.

Plou and co-workers have described the preparation of maltotriose fatty acid monoesters and their surface activity [8]; however, the



1 : R = R' = H (raffinose)
1a: R = $CO(CH_2)_nCH_3$, R' = H (6-*O*-acylraffinose)
1b: R = H, R' = $CO(CH_2)_nCH_3$ (1"-*O*-acylraffinose)



2 : R = R' = H (melezitose)
2a: R = $CO(CH_2)_nCH_3$, R' = H (6-*O*-acylmelezitose)
2b: R = H, R' = $CO(CH_2)_nCH_3$ (6"-*O*-acylmelezitose)

Fig. 1. Structure of the non-reducing trisaccharides raffinose (**1**), melezitose (**2**), and their fatty acid monoesters prepared by enzymatic synthesis.

authors did not explore their use as detergents in biochemistry. At the same time, these maltotriose derivatives are present as anomeric mixtures, whereas the non-reducing trisaccharides derivatives presented here avoid this problem.

3.2. CMC determination

The estimation of the CMCs for the compounds in the three different homologous series was carried out by a colorimetric dye-inclusion assay previously developed for the determination of the CMC of non-ionic surfactants [27] including sugar-based surfactants [18]. The method is based on the dependence of the extinction coefficient of Coomassie brilliant blue G-250 at 620 nm with the hydrophobicity of its environment.

Fig. 2 shows the corresponding curves for the CMC determination of the compounds in the homologous series of 6-*O*-acylraffinose, and Table 2 summarizes the values obtained for all compounds. The three families of monoesters bearing saturated acyl chains of 10, 12, 14, 16, and 18 carbon atoms have CMC values covering a wide range from mM to μM. As expected, the longer the acyl chain, the lower the CMC [28].

Differences in the CMC values of different regioisomers of sucrose fatty acid monoesters have been reported: the CMC of 1′-*O*-tetradecanoyl-sucrose and 6-*O*-tetradecanoyl sucrose were 9.1×10^{-5} and 1.3×10^{-4} M, respectively, while the CMC of 6-*O*-dodecanoyl-sucrose was 2.6 times greater than 1′-*O*-dodecanoyl-sucrose [21]. In the case of the raffinose derivatives described here, an influence of the acylation position on the micellization properties was also observed. For example, the CMC values obtained for 6-*O*-decanoylraffinose were 2.2 times greater than for 1″-*O*-decanoylraffinose. This behavior is completely analogous to the one described for the pair 6-*O*-acylsucrose/1′-*O*-acylsucrose [21]. It is possible that the origin is the same in both cases since raffinose is a 6-*O*-α-galactopyranosylsucrose. In fact, at the micelle surface, the packing of the carbohydrate polar heads could be different depending on the particular regioisomer, generating a different CMC in each case. It is logical to think that such packing will depend on the conformation of the carbohydrate in the monoester. Acylation of the OH-1 of the fructofuranosyl residue results in the breaking of the intramolecular hydrogen bond $HO\text{-}2_{glucose}\cdots HO\text{-}1_{fructose}$ present in both sucrose and raffinose [45], originating as a consequence differences in the carbohydrate conformation among regioisomers [46].

Additional dynamic light scattering measurements for detergents of the 6-*O*-acylraffinose family suggest a spherical micellar shape, whereas the estimation of the aggregation numbers shows the expected increase upon increasing the length the alkyl chain (see Table S1 in Supplementary data).

Table 2
Estimated CMC of the prepared surfactants and calculated HLB values.

Compound[a]	Abbreviation	Chain length	HLB[b]	CMC[c] (g/l)	CMC (μM)
6-*O*-decanoylraffinose	R6D	C10	15.3	1.8	2733
1″-*O*-decanoylraffinose	R1D	C10	15.3	0.8	1214
Decanoylmelezitose	MD	C10	15.3	1.1	1670
6-*O*-lauroylraffinose	R6L	C12	14.7	0.22	320
1″-*O*-lauroylraffinose	R1L	C12	14.7	0.16	233
Lauroylmelezitose	ML	C12	14.7	0.17	247
6-*O*-myristoylraffinose	R6M	C14	14.1	0.036	50
1″-*O*-myristoylraffinose	R1M	C14	14.1	0.032	45
Myristoylmelezitose	MM	C14	14.1	0.026	36
6-*O*-palmitoylraffinose	R6P	C16	13.6	0.014	19
1″-*O*- palmitoylraffinose	R1P	C16	13.6	0.010	13
Palmitoylmelezitose	MP	C16	13.6	0.008	11
6-*O*-stearoylraffinose	R6S	C18	13.1	0.007	9
1″-*O*-stearoylraffinose	R1S	C18	13.1	0.007	9
Stearoylmelezitose	MS	C18	13.1	0.006	8

[a] Each acylmelezitose is a mixture ca. 2:1 of the 6-*O*-acyl/6″-*O*-acyl derivatives.
[b] HLB = 20 (molecular weight hydrophilic group)/(molecular weight of the surfactant).
[c] CMC for control detergents: CHAPS (1.8 g/l; 2.3 mM), DDM (0.06 g/l; 116 μM), SUL (0.085 g/l; 162 μM).

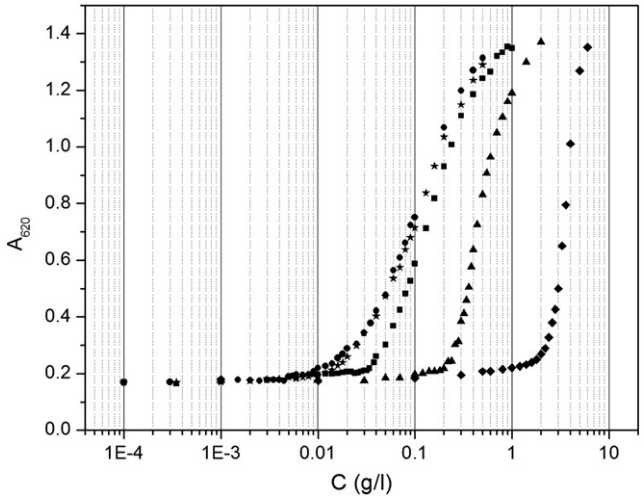


Fig. 2. CMC estimation of the 6-*O*-acylraffinose homologous series. Increasing concentrations of R6D (♦), R6L (▲), R6M (■), R6P (★), and R6S (●) were mixed with the dye as described in the Materials and methods section and the absorbance was measured at 620 nm.

3.3. Preliminary test of vesicle solubilization by 6-*O*-lauroylraffinose

To confirm the detergent ability of the prepared trisaccharide monoesters, a preliminary test with 6-*O*-lauroylraffinose (R6L) was carried out to check its capacity to solubilize phospholipid vesicles. It is well established that turbidity measurements constitute a very convenient and powerful technique for the quantitative analysis of liposome solubilization by detergents [30]. Fig. 3 depicts the changes in turbidity of an aqueous suspension of phosphatidylcholine (PC) liposomes (1 mg of phospholipid/ml, i.e., 1.32 mM) as a function of detergent concentration. The results obtained follow the typical three-stage model for the solubilitation process [47,48]: stage I, which involves partitioning of detergent monomers between the aqueous medium and the lipid bilayer until liposomes are saturated with detergent (Sat); stage II, where detergent addition promotes gradual liposome solubilization resulting in the coexistence of lipid–detergent mixed micelles and lipid bilayers saturated with detergent; and final stage III, which is characterized by complete solubilization of lipid (Sol) into small mixed micelles with the suspension becoming optically transparent. The overall pattern of the curve is similar to that observed for the solubilization of large unilamellar vesicles by OG [30]. This preliminary test confirmed the capacity of a representative member of these homologous series to solubilize phospholipid vesicles. It was thus expected that the new detergents could likewise solubilize native cell membranes.

3.4. Screening of the new detergents for solubilizing/extraction capacity of an integral membrane protein under native conditions

The usefulness of the prepared detergents in membrane biochemistry ultimately depends on their capacity to extract and solubilize membrane proteins under native conditions. The behavior of the new detergents was studied by solubilization screening of a model integral membrane protein, BmrA, a dimeric ABC multidrug transporter from *B. subtilis* where each monomer is comprised by 6 transmembrane segments [22,23]. ABC transporters are transmembrane proteins that utilize the energy of ATP hydrolysis to transport a wide variety of substrates, sometimes including therapeutic drugs, across membranes [49].

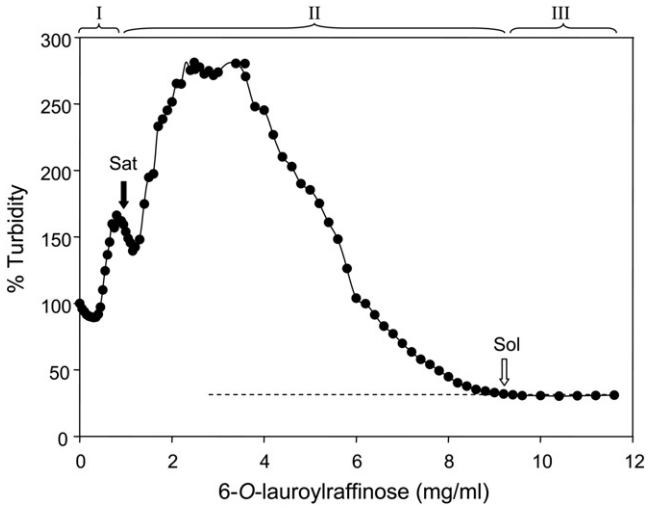


Fig. 3. Liposomes solubilization by 6-*O*-lauroylrafinose. Detergent was added to the liposome suspension under constant stirring at room temperature, and the turbidity was measured at 400 nm after detergent equilibration. 100% Turbidity correspond to the one observed for the liposome suspension in the absence of detergent. I: Stage I; II: Stage II; III: Stage III; Sat: minimal detergent concentration required to saturate the lipid bilayer; Sol: minimal detergent concentration required to completely solubilize the liposomes.

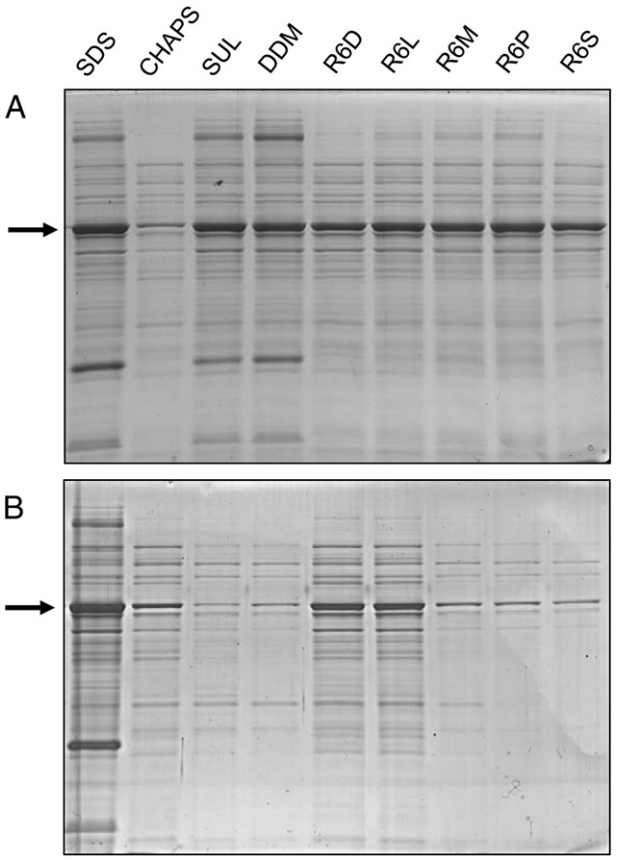


Fig. 4. Solubilization screening by SDS-PAGE of novel detergents from the 6-*O*-acylraffinose family in comparison with commercially available detergents SDS, CHAPS, SUL, and DDM. Detergent concentration was 1% (A) or 10 x CMC (B). BmrA band (ca. 66 kDa) is indicated by an arrow. 4 μl of supernatant was charged per well, and the gel was stained with Coomassie brilliant blue.

Solubilizing/Extraction capacity of the new detergents was compared with those observed for other typically employed detergents in membrane protein research: SDS as a strong denaturing anionic detergent (control of complete solubilization), CHAPS as a mild zwitterionic detergent, and two non-ionic carbohydrate-based detergents, dodecylmaltoside (DDM), which is the detergent employed in the original solubilization and purification of the model protein [22,24,34], and sucrose monolaurate (SUL), which is also a carbohydrate fatty acid monoester. *E. coli* membranes containing overexpressed His-tagged BmrA (ca. 66 kDa) were isolated as previously described [23]. Two different concentrations of each detergent were employed in the solubilization screening: 1% as previously described for the extraction of BmrA from *E. coli* plasma membranes using DDM [22,24,34] and 10× CMC for each compound as has already been used in previous detergent screens for membrane proteins [50,51]. Analysis by SDS-PAGE of the supernatant obtained after ultracentrifugation of the mixture containing the detergent solubilized membranes provides a convenient assay of the solubilizing/extractive capacity of the different detergents. The results for the 6-*O*-acylraffinose family are shown in Fig. 4. Considering the SDS lane as a positive control, it can be clearly observed that at 1% concentration (Fig. 4A), the different members of the family solubilize the target protein at a similar extent when compared to each other and to the commercial DDM and SUL. Of all the detergent tested, CHAPS displays the poorest solubilizing performance at this concentration. On the other hand, at 10× CMC (Fig. 4B), the best detergents were 6-*O*-decanoylraffinose (R6D) and 6-*O*-lauroylraffinose (R6L) while the other members in the series 6-*O*-myristoylraffinose (R6M), 6-*O*-palmitoylraffinose (R6P), and 6-*O*-stearoylraffinose (R6S) display a similar or slightly better solubilizing capacity than DDM and SUL. It is well known that the longer the alkyl chain in a non-ionic detergent homologous series, the lower the CMC and the higher the aggregation number (number of detergent molecules per micelle) [28]. The reason the members in the series with a higher CMC, R6D and R6L, have better solubilizing capacity at this concentration (10× CMC) than the other members with longer alkyl chain is related to the fact that there is a larger number of micelles in solution for these two detergents at 10× CMC than for the others due to their lower aggregation number. Since membrane protein extraction is very dependent – assuming an important contribution of solubilization by micellar attack – on the number of micelles present for detergents with highly hydrophilic heads as it has been described for DDM [11,52], it is not surprising that the shorter chain members in the series display a better solubilizing/extraction capacity at 10× CMC. For the other two families, 1″-*O*-acylraffinose and monoacylmelezitose, extraction of BmrA was similar to that observed for the family 6-*O*-acylraffinose (see Fig. S2 and Fig. S3 in Supplementary data). Protein concentration was also measured in the supernatants obtained with 1% and 10× CMC concentration of detergents and compared with the one obtained for SDS, which was taken as reference of complete membrane solubilization and protein extraction, confirming the gel electrophoresis results (see Fig. S4 in Supplementary data).

The nondenaturing property of the new detergents was tested by measuring the native ATPase activity of BmrA in the supernatants obtained in the previous solubilization screening. The results obtained for the 6-*O*-acylraffinose family in comparison with DDM as control are shown in Fig. 5. Interestingly, all derivatives except the stearoyl monoester yield higher enzymatic activity than the DDM control. Within this family, the shorter chain derivatives C10 and C12 yield much higher activity than the longer chain ones (C14, C16, and C18) and DDM. A similar pattern was observed for the other two homologous series (see Fig. S5 in Supplementary data), likewise the structurally analogous SUL yields slightly lower ATPase activity than DDM.

The potential of R6L as a detergent was also evaluated analyzing its ability to solubilize other integral membrane proteins of biomedical interest in different lipidic context, i.e., membranes with different protein:lipid ratio and different lipids and sterol composition. Fig. 6 shows that R6L was also efficient in solubilizing (i) mammalian Pgp/MDR1/ABCB1, an ABC multidrug transporter with 12 transmembrane segments overexpressed in tumor cells [53]; (ii) *Leishmania* LPgp/

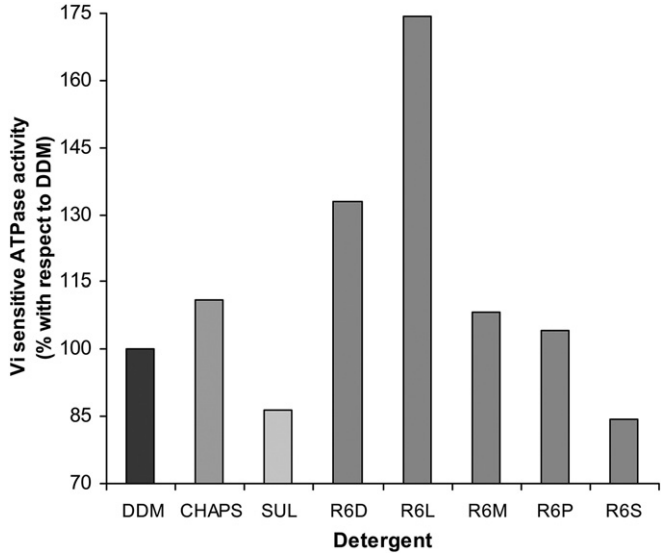


Fig. 5. Vanadate-sensitive ATPase activity in the supernatants obtained in the solubilization screening with the 6-*O*-acylraffinose family. Detergent concentration was kept at the CMC of each detergent for the enzymatic assay. Activity measured for DDM (78 ± 6 nmol Pi/min per mg total protein) was assigned a reference value of 100%. Data are the media of two independent experiments performed in triplicate with standard deviation below 10%.

LMDR1/LABCB5, another ABC transporter with 12 transmembrane segments overexpressed in a multidrug resistant line of the protozoan parasite *Leishmania tropica* conferring miltefosine resistance [32,54]; and (iii) *Leishmania* Ros3, a plasma membrane protein with two transmembrane segments constitutively expressed in wild-type *L. tropica* whose mutation confers miltefosine resistance [55].

Thus it can be concluded that some of the novel detergents display a high solubilizing/extraction capacity of the model intrinsic membrane proteins under nondenaturing conditions preserving their native ATPase activity. The best performing detergent in overall terms of amount of extracted protein and preservation of enzymatic activity was 6-*O*-lauroylraffinose (R6L) and for this reason was selected for the following steps of purification and reconstitution of the model protein. In addition, R6L has the same lipophilic chain length (C12) as DDM – the detergent previously employed for BmrA purification and reconstitution [22,24,34] – and is regioisomerically pure (as the whole 6-*O*-acylraffinose family). Such regioisomeric purity is very important for issues related to membrane protein crystallization [16,17], as well as for the enzymatic procedure of proteoliposome reconstitution later described (Section 3.6).

3.5. Purification of BmrA with a selected detergent

In this stage, purification of the model membrane protein BmrA (which is fused to a C-terminal 6-His tag) was carried out in a similar way as has been described using DDM [22] but in our case employing the selected detergent, R6L, from the previous screening study instead of DDM.

First, BmrA was solubilized in 1% R6L from the *E. coli* plasma membranes (see Materials and methods). The supernatant obtained after ultracentrifugation of the solubilized membranes was purified by affinity chromatography using a batch method with Ni-NTA (nickel-nitrilotriacetic acid) agarose including 0.05% R6L in the loading and eluting buffers. Fractions from the gravity column were analyzed by SDS-PAGE (Fig. 7). Pure fractions were combined, and imidazole was removed using a desalting column (including 0.05% R6L in the washing and eluting buffers). ATPase activity of purified BmrA in 0.05% R6L was 1.1 ± 0.2 μmol Pi/min per mg protein at 37 °C, similar to the reported value for the same purified protein in 0.05% DDM [56], suggesting that in both detergents, the protein is present in the same functionally active dimeric form [56]. In summary, the purification conditions of BmrA using DDM as detergent were compatible with the use of R6L as alternative, validating this detergent also for Ni-NTA affinity chromatographic purification of membrane proteins.

3.6. Enzymatic preparation of (proteo)liposomes

Phospholipid vesicles incorporating purified membrane proteins (proteoliposomes) are a powerful tool for elucidating both functional and structural aspects of these kinds of proteins [48]. Of the available strategies for reconstitution of membrane proteins into liposomes, probably the most popular due to its robustness and reproducibility is the detergent-mediated approach [57]. The reconstitution process involves the comicellization of the purified protein in an excess of lipids and detergent, to form a solution of mixed lipid–protein–detergent and lipid–detergent micelles. Removal of the detergent from these micellar solutions results in the progressive formation of closed lipid bilayers into which the protein eventually incorporates [57].

Of the different techniques available for detergent removal, the most appropriate one for detergents of low CMC that are not readily removed by dialysis, like DDM, is the physical adsorption of the detergent on the surface of porous hydrophobic polystyrene bead

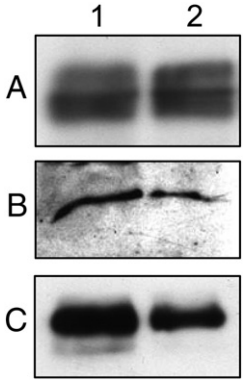


Fig. 6. Solubilization of different membrane proteins with R6L monitored by Western blot. 15 μl of membranes (20 μg of protein) (lane 1) or the same volume of solubilized supernatant (lane 2) was loaded in each well. (A) Total membranes from a *L. tropica* MDR line revealed with antiLMDR1 antibody (dilution 1:1000). (B) Total membranes from a *L. tropica* wild-type line revealed with antiLRos3 antibody (dilution 1:3000). (C) Total membranes from a mammal MDR line revealed with JSB-1 antibody (abcam) (dilution 1:200).

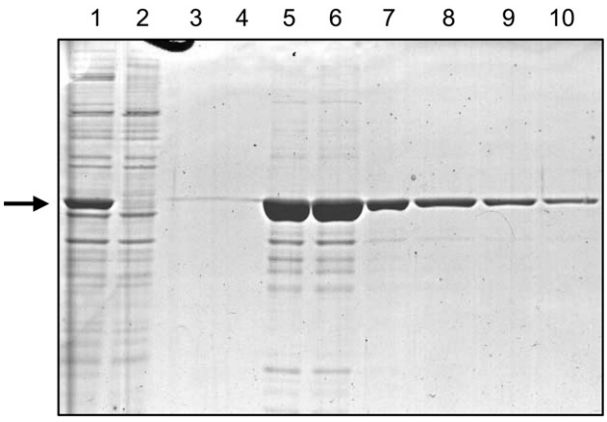


Fig. 7. SDS-PAGE analysis of BmrA (ca. 66 kDa) purification by Ni-NTA affinity chromatography. Lane 1, loaded supernatant. Lane 2, unbound proteins. Lanes 3–4, flow-through. Lanes 5–10: protein eluted with 250 mM imidazole. Gel was stained with Coomassie brilliant blue. BmrA band (ca. 66 kDa) is indicated by an arrow.

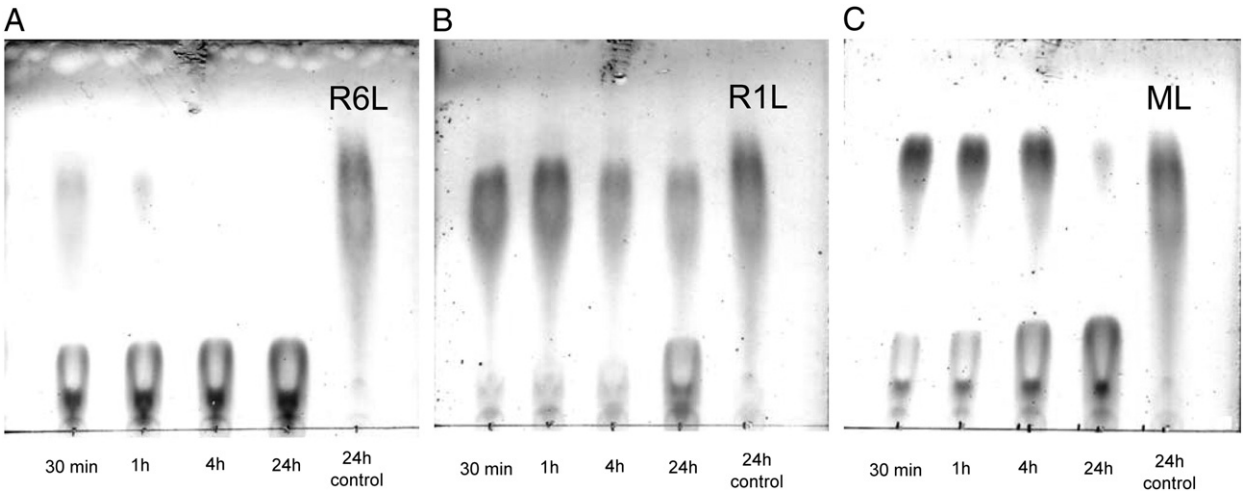


Fig. 8. TLC kinetic analysis of the enzymatic hydrolysis of detergents. R6L (A), R1L (B), and ML (C) were incubated at room temperature with the lipase form *T. lanuginosus* covalently immobilized on Eupergit C as described in the Materials and methods section, and the hydrolisis of the ester bond was monitored by TLC.

resins such as Bio-Beads SM2 [58]. This in fact has been the technique employed to remove DDM for the preparation of proteoliposomes containing purified BmrA [34].

Another possible alternative to physically extracting the surfactant (as with the Bio-Beads) is the use of enzymes to convert the surfactant into molecules that are no longer surface active. The enzymatic reaction is chosen to hydrolyze the covalent link between the hydrophobic and hydrophilic moieties of the detergent molecule. The lipid-solubilizing power of the detergent is progressively decreased, leading to the self-formation of vesicles. This strategy, like the use of Bio-Beads, allows for control of the rate of detergent removal (thus the rate of proteoliposome formation), and it has been applied for the preparation of vesicles starting from mixed lipid–DDM micelles and subsequent hydrolysis of the detergent with a glycosidase [59,60]. A similar procedure has been reported to prepare vesicles starting from mixed micelles of lipids and 6-*O*-octanoylglucose or 6-*O*-octanoylmannose and hydrolyzing the sugar fatty acid ester with an immobilized lipase to produce spontaneous liposome formation [36].

We decided to study the enzyme-mediated preparation of (proteo)liposomes with our detergents employing *T. lanuginosus* lipase covalently immobilized onto Eupergit C [35,61] to cleave the ester linkage and compare it also with the vesicles obtained by detergent removal with Bio-Beads SM-2. The principle of reversibility suggests that the same enzyme employed to catalyze the sugar ester formation in organic media should be able to hydrolyze such ester under aqueous buffer conditions.

Previously reported conditions for the lipase-catalyzed hydrolysis of 6-*O*-octanoylglucose [36] were extrapolated to the hydrolysis of the 6-*O*-lauroylraffinose (R6L), 1″-*O*-lauroylraffinose (R1L), and melezitose monolaurate (ML) using *T. lanuginosus*-Eupergit C lipase and monitoring the reaction by TLC (Fig. 8). Hydrolysis of R1L was much slower than the regioisomer R6L indicating that the enzyme keeps the same regioselectivity observed for the synthesis of trisaccharide monoesters in organic media [19]. Likewise, although melezitose monolaurate (ML) was synthesized in organic media using this lipase, the rate of hydrolysis is much slower than with R6L, which actually matched with the lower yield of transesterification of melezitose (compared with raffinose) when using this lipase in organic media and vinyl laurate as acylating agent [19].

Next, we approached the preparation of liposomes using the lipase-mediated strategy and compared it with the Bio-Beads SM2 strategy. In a first stage, we compared the behavior of DDM and R6L for preparing vesicles using mixed micelles containing the detergent and *E. coli* phospholipids (total extract) and successive additions of Bio-Beads SM2 to remove the detergent as previously described [34]. Characterization of liposome size by dynamic light scattering (see Materials and methods) showed a very similar size in both cases (Fig. 9A), with an average diameter of 180 nm in the case of DDM – in agreement with reported results [62] – and 170 nm in the case of R6L, which indicates a similar removal rate of both detergents since the size of vesicles prepared by this method are dependent on the rate at which the detergent is removed [58]. These results validate R6L as a compatible detergent for the preparation of liposomes using the Bio-Beads SM2 strategy. Then we prepared liposomes using the same mixed micelles and hydrolyzing the detergent R6L with the lipase from *T. lanuginosus* immobilized on Eupergit C (see Materials and methods). Interestingly, the measured size of the formed liposomes by DLS was much larger this time with an average diameter of 550 nm (Fig. 9A). The larger size of the vesicle formed by lipase-mediated hydrolysis of R6L in comparison with that obtained using Bio-Beads SM2 removal of the detergent must be associated with a slower elimination rate of the detergent. In fact, TLC analysis of R6L and DDM removal by Bio-Beads SM2 using the standard conditions described by Jault and co-workers [34] confirmed a faster detergent disappearance than the hydrolysis rate observed when using the enzyme (data not shown). Lamellarity of the formed liposomes was determined by a NBD–lipid fluorescence dithionite reduction assay [38] showing the

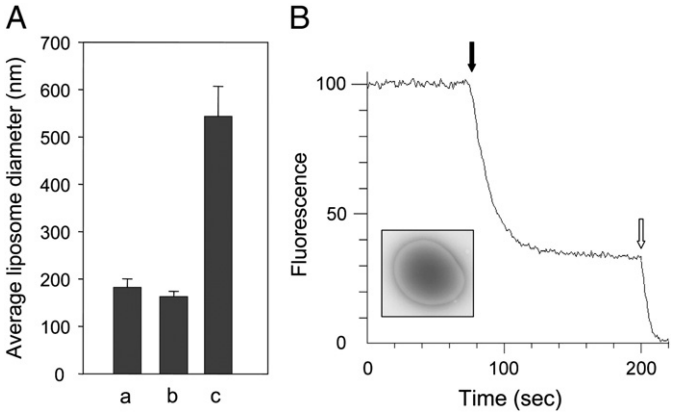


Fig. 9. Liposomes characterization. A. Liposomes size as determined by DLS for vesicles prepared by DDM (a) or R6L (b) removal using Bio-Beads SM2 or by hydrolysis of R6L with the immobilized lipase (c). B. Lamellarity of liposomes prepared with the enzymatic method determined by monitoring the quenching of (palmitoyl, C_6-NBD)-PC fluorescence after dithionite addition (black arrow) and detergent addition (white arrow) and by transmission electron microscopy (inset).

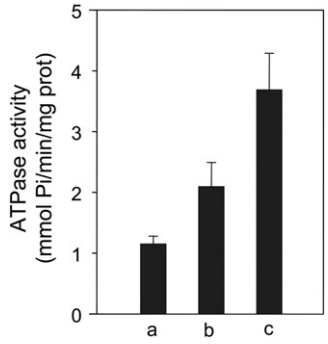


Fig. 10. ATPase activity of purified and reconstituted BmrA. The ATPase activity of purified BmrA was measured in the presence of R6L (a), R6L and lipids (b), and after reconstitution in proteoliposomes by the lipase-mediated method (c). Data are the means ± SD of two independent experiments performed in triplicate.

population to be unilamellar (Fig. 9B) and this was confirmed by transmission electron microscopy (inset in Fig. 9B).

Finally, we probed this methodology for the preparation of proteoliposomes containing purified membrane protein BmrA in order to confirm the compatibility of the lipase-mediated approach as a reconstitution method. After proteoliposome preparation, the native enzymatic activity was measured in the proteoliposomes and compared with the activity observed for the purified protein in the buffer containing R6L and the purified protein after addition of lipids, since lipid addition to purified ABC transporters stimulates their activity [63,64]. The results summarized in Fig. 10 clearly indicate that protein reconstitution into proteoliposomes is the most efficient method to regain a high ATPase activity. In fact we obtained an activity similar to the reported value for BmrA reconstituted in proteoliposomes after removal of DDM with Bio-Beads [22], suggesting the same functionally active oligomerization state (homodimer) of the protein [65]. Likewise, as described by Jault and co-workers [22,65], the high ATPase activity indicates that the enzymatic reconstitution protocol seems to favor the "inside–out" orientation of BmrA in the proteoliposomes. These results suggest that the lipase-mediated approach to reconstitute BmrA into proteoliposomes here described is suitable in general for functional studies of membrane proteins extracted and purified with these novel detergents.

4. Conclusions

In summary, various homologous series of non-reducing trisaccharide fatty acid monoesters have been prepared by enzyme-catalyzed acylations in organic solvents. A preliminary physicochemical characterization of these surfactants has been carried out in order to determine their CMCs, which cover a broad range; likewise their capacity to dissolve phospholipid bilayers has been proved. The usefulness of the prepared detergents in membrane protein research has been studied in a comparative screening test for solubilizing/extraction capacity of a model integral membrane protein (the ABC transporter BmrA overexpressed in *E. coli*) under native conditions showing an excellent behavior. The overall performance of the new detergents was similar to other commercial carbohydrate-based detergents. Interestingly, several trisaccharide monolaurates were even better detergents than the most typically employed DDM. 6-*O*-Lauroylraffinose was also efficient at extracting other membrane proteins from different lipidic environments. The novel detergents are likewise compatible with common membrane protein purification techniques such as Ni-NTA affinity chromatography. Finally, these compounds may be used for the preparation of (proteo)liposomes by the detergent removal approach, not only using the classical detergent adsorption on hydrophobic resins like Bio-Beads but also by enzyme-catalyzed hydrolysis of the ester bond using a covalently immobilized version of the same lipase employed for the corresponding surfactant synthesis in organic solvents.

Altogether, these results show the new detergents as promising tools to expand the arsenal for membrane protein studies.

Acknowledgments

We are grateful to Dr. Jean-Michel Jault (Institut de Biologie Structurale, Grenoble, France) for providing the construction with the BmrA gene used in this article. We also thank Joan Estelrich and Sonia García-Jimeno for preparing the PC liposomes for our test of vesicles solubilization and Dr. Craig Mckibbin for providing language help.

This work was supported by the Spanish Grants MSC-FIS PI081902 and MCI-PII-200820I212 (to J.M.P.V.), ISCIII-Red de Investigación Cooperativa en Enfermedades Tropicales (RICET) RD06/0021/0002 (to F.G.), European Reintegration Grant MERG-CT-2004-513607 (to J.M.P.V and F.G.), and Plan Andaluz de Investigación (Cod. BIO130). S. R.-V. is grateful to "MICINN" (Projects: MAT2009-13155-C04-02, and 03) and "Junta de Andalucía" (Project: P07-FQM-02496) for financial support. J.C.M. is grateful to "MICINN" (Project: CTQ2009-13705).

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2010.11.031.

References

[1] C. Carnero Ruiz (Ed.), Sugar-Based Surfactants: Fundamentals and Applications, CRC Press, Boca Raton, Florida, 2008.
[2] K. Hill, O. Rhode, Sugar-based surfactants for consumer products and technical applications, Fett - Lipid 101 (1999) 25–33.
[3] L. Osipow, Methods of preparation of fatty acid esters of sucrose, Ind. Eng. Chem. 48 (1956) 1459–1462.
[4] M.H. Moh, T.S. Tang, G.H. Tan, Improved separation of sucrose ester isomers using gradient high performance liquid chromatography with evaporative light scattering detection, Food Chem. 69 (2000) 105–110.
[5] I. Pérez-Victoria, A. Zafra, J.C. Morales, Determination of regioisomeric distribution in carbohydrate fatty acid monoesters by LC-ESI-MS, Carbohydr. Res. 342 (2007) 236–242.
[6] F.J. Plou, M.A. Cruces, M. Ferrer, G. Fuentes, E. Pastor, M. Bernabe, M. Christensen, F. Comelles, J.L. Parra, A. Ballesteros, Enzymatic acylation of di- and trisaccharides with fatty acids: choosing the appropriate enzyme, support and solvent, J. Biotechnol. 96 (2002) 55–66.
[7] N. Becerra, C. Toro, A.L. Zanocco, E. Lemp, G. Gunther, Characterization of micelles formed by sucrose 6-*O*-monoesters, Colloid Surf. Physicochem. Eng. Asp. 327 (2008) 134–139.
[8] M. Ferrer, F. Comelles, F.J. Plou, M.A. Cruces, G. Fuentes, J.L. Parra, A. Ballesteros, Comparative surface activities of di- and trisaccharide fatty acid esters, Langmuir 18 (2002) 667–673.
[9] G. Garofalakis, B.S. Murray, D.B. Sarney, Surface activity and critical aggregation concentration of pure sugar esters with different sugar headgroups, J. Colloid Interface Sci. 229 (2000) 391–398.
[10] V. Molinier, P.H.J. Kouwer, J. Fitremann, A. Bouchu, G. Mackenzie, Y. Queneau, J.W. Goodby, Self-organizing properties of monosubstituted sucrose fatty acid esters: the effects of chain length and unsaturation, Chem. Eur. J. 12 (2006) 3547–3557.
[11] M. le Maire, P. Champeil, J.V. Moller, Interaction of membrane proteins and lipids with solubilizing detergents, Biochim. Biophys. Acta Biomembr. 1508 (2000) 86–111.
[12] G.G. Prive, Detergents for the stabilization and crystallization of membrane proteins, Methods 41 (2007) 388–397.
[13] Y. Gohon, J.-L. Popot, Membrane protein–surfactant complexes, Curr. Opin. Colloid Interface Sci. 8 (2003) 15–22.
[14] T. Vanaken, S. Foxall-Vanaken, S. Castleman, S. Ferguson-Miller, F. Sidney Fleischer, Becca, Alkyl glycoside detergents: synthesis and applications to the study of membrane proteins, Methods in Enzymology, Vol. 125, Academic Press, 1986, pp. 27–35.
[15] D. Abran, F. Boucher, T. Hamanaka, K. Hiraki, Y. Kito, K. Koyama, R.M. Leblanc, H. Machida, G. Munger, M. Seidou, M. Tessier, On some physicochemical properties of sucrose esters and the stability they confer to membrane proteins, J. Colloid Interface Sci. 128 (1989) 230–236.
[16] R.M. Garavito, D. Picot, The art of crystallizing membrane proteins, Methods 1 (1990) 57–69.
[17] H. Zulauf, Detergent phenomena in membrane protein crystallization, in: H. Michel (Ed.), Crystallization of Membrane Proteins, CRC Press, Boca Raton, Florida, 1991, pp. 53–72.

[18] L. Greffe, L. Bessueille, V. Bulone, H. Brumer, Synthesis, preliminary characterization, and application of novel surfactants from highly branched xyloglucan oligosaccharides, Glycobiology 15 (2005) 437–445.
[19] I. Pérez-Victoria, J.C. Morales, Complementary regioselective esterification of non-reducing oligosaccharides catalyzed by different hydrolases, Tetrahedron 62 (2006) 878–886.
[20] I. Pérez-Victoria, J.C. Morales, One- and two-step enzymatic synthesis of polymerizable vinyladipoyl mono- and diesters of non-reducing trisaccharides, J. Mol. Catal. B Enzym. 48 (2007) 8–15.
[21] T. Polat, R. Linhardt, Syntheses and applications of sucrose-based esters, J. Surfactants Deterg. 4 (2001) 415–421.
[22] E. Steinfels, C. Orelle, J.-R. Fantino, O. Dalmas, J.-L. Rigaud, F. Denizot, A. Di Pietro, J.-M. Jault, Characterization of YvcC (BmrA), a multidrug ABC transporter constitutively expressed in *Bacillus subtilis*, Biochemistry 43 (2004) 7491–7502.
[23] E. Steinfels, C. Orelle, O. Dalmas, F. Penin, B. Miroux, A. Di Pietro, J.-M. Jault, Highly efficient over-production in *E. coli* of YvcC, a multidrug-like ATP-binding cassette transporter from *Bacillus subtilis*, Biochim. Biophys. Acta BBA Biomembr. 1565 (2002) 1–5.
[24] M. Chami, E. Steinfels, C. Orelle, J.-M. Jault, A. Di Pietro, J.-L. Rigaud, S. Marco, Three-dimensional structure by cryo-electron microscopy of YvcC, an homo-dimeric ATP-binding cassette transporter from *Bacillus subtilis*, J. Mol. Biol. 315 (2002) 1075–1085.
[25] I. Pérez-Victoria, J.C. Morales, Regioselectivity in acylation of oligosaccharides catalyzed by the metalloprotease thermolysin, Tetrahedron 62 (2006) 2361–2369.
[26] I. Pérez-Victoria, A. Zafra, J.C. Morales, Positive-ion ESI mass spectrometry of regioisomeric nonreducing oligosaccharide fatty acid monoesters: in-source fragmentation of sodium adducts, J. Mass Spectrom. 43 (2008) 633–638.
[27] K.S. Rosenthal, F. Koussaie, Critical micelle concentration determination of non-ionic detergents with Coomassie brilliant blue G-250, Anal. Chem. 55 (1983) 1115–1117.
[28] J.M. Neugebauer, P.D. Murray, Detergents: an overview, Methods in Enzymology, Vol. 182, Academic Press, 1990, pp. 239–253.
[29] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, Biochim. Biophys. Acta BBA Biomembr. 812 (1985) 55–65.
[30] M.T. Paternostre, M. Roux, J.L. Rigaud, Mechanisms of membrane protein insertion into liposomes during reconstitution procedures involving the use of detergents. 1. Solubilization of large unilamellar liposomes (prepared by reverse-phase evaporation) by Triton X-100, octyl glucoside, and sodium cholate, Biochemistry 27 (1988) 2668–2677.
[31] S. Chifflet, A. Torriglia, R. Chiesa, S. Tolosa, A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: application to lens ATPases, Anal. Biochem. 168 (1988) 1–4.
[32] M.J. Chiquero, J.M. Pérez-Victoria, F. O'Valle, J.M. González-Ros, R.G. del Moral, J.A. Ferragut, S. Castanys, F. Gamarro, Altered drug membrane permeability in a multidrug-resistant *Leishmania tropica* line, Biochem. Pharmacol. 55 (1998) 131–139.
[33] F. Muñoz-Martínez, P. Lu, F. Cortes-Selva, J.M. Pérez-Victoria, I.A. Jiménez, A.G. Ravelo, F.J. Sharom, F. Gamarro, S. Castanys, Celastraceae Sesquiterpenes as a new class of modulators that bind specifically to human P-glycoprotein and reverse cellular multidrug resistance, Cancer Res. 64 (2004) 7130–7138.
[34] C.d. Orelle, O. Dalmas, P. Gros, A. Di Pietro, J.-M. Jault, The conserved glutamate residue adjacent to the Walker-B motif is the catalytic base for ATP hydrolysis in the ATP-binding cassette transporter BmrA, J. Biol. Chem. 278 (2003) 47002–47008.
[35] M. Ferrer, F.J. Plou, G. Fuentes, M.A. Cruces, L. Andersen, O. Kirk, M. Christensen, A. Ballesteros, Effect of the immobilization method of lipase from *Thermomyces lanuginosus* on sucrose acylation, Biocatal. Biotransform. 20 (2002) 63–71.
[36] H. Peters, C. Schmidt-Dannert, L. Cao, U.T. Bornscheuer, R.D. Schmid, Purification and reconstitution of an integral membrane protein, the photoreaction center of Rhodobacter sphaeroides, using synthetic sugar esters, Biotechniques 28 (2000) 1214–1219.
[37] D.E. Koppel, Analysis of macromolecular polydispersity in intensity correlation spectroscopy: the method of cumulants, J. Chem. Phys. 57 (1972) 4814–4820.
[38] J.C. McIntyre, R.G. Sleight, Fluorescence assay for phospholipid membrane asymmetry, Biochemistry 30 (1991) 11819–11827.
[39] W.C. Griffin, Classification of surface-active agents by HLB, J. Soc. Cosmet. Chem. 1 (1949) 311–326.
[40] W.C. Griffin, Calculation of HLB values of non-ionic surfactants, J. Soc. Cosmet. Chem. 5 (1954) 259–267.
[41] M. Ferrer, M.A. Cruces, F.J. Plou, M. Bernabe, A. Ballesteros, A simple procedure for the regioselective synthesis of fatty acid esters of maltose, leucrose, maltotriose and *n*-dodecyl maltosides, Tetrahedron 56 (2000) 4053–4061.
[42] G. Carrea, S. Riva, F. Secundo, B. Danieli, Enzymatic-synthesis of various 1′-*O*-sucrose and 1-*O*-fructose esters, J. Chem. Soc. Perkin Trans. 1 (1989) 1057–1061.
[43] J.O. Rich, B.A. Bedell, J.S. Dordick, Controlling enzyme-catalyzed regioselectivity in sugar ester synthesis, Biotechnol. Bioeng. 45 (1995) 426–434.
[44] S. Soultani, S. Ognier, J.-M. Engasser, M. Ghoul, Comparative study of some surface active properties of fructose esters and commercial sucrose esters, Colloids Surf. A 227 (2003) 35–44.
[45] D.B. Davies, J.C. Christofides, Comparison of intramolecular hydrogen-bonding conformations of sucrose-containing oligosaccharides in solution and the solid-state, Carbohydr. Res. 163 (1987) 269–274.
[46] V. Molinier, B. Fenet, J. Fitremann, A. Bouchu, Y. Queneau, PFGSE-NMR study of the self-diffusion of sucrose fatty acid monoesters in water, J. Colloid Interface Sci. 286 (2005) 360–368.
[47] D. Lichtenberg, R.J. Robson, E.A. Dennis, Solubilization of phospholipids by detergents structural and kinetic aspects, Biochim. Biophys. Acta BBA Rev. Biomembr. 737 (1983) 285–304.
[48] J.-L. Rigaud, B. Pitard, D. Levy, Reconstitution of membrane proteins into liposomes: application to energy-transducing membrane proteins, Biochim. Biophys. Acta BBA Bioenerg. 1231 (1995) 223–246.
[49] C.F. Higgins, ABC transporters: from microorganisms to man, Annu. Rev. Cell Biol. 8 (1992) 67–113.
[50] A.K. Mohanty, M.C. Wiener, Membrane protein expression and production: effects of polyhistidine tag length and position, Protein Expr. Purif. 33 (2004) 311–325.
[51] M.C. Wiener, A pedestrian guide to membrane protein crystallization, Methods 34 (2004) 364–372.
[52] U. Kragh-Hansen, M. le Maire, J.V. Møller, The mechanism of detergent solubilization of liposomes and protein-containing membranes, Biophys. J. 75 (1998) 2932–2946.
[53] F.J. Sharom, ABC multidrug transporters: structure, function and role in chemoresistance, Pharmacogenomics 9 (2008) 105–127.
[54] J.M. Pérez-Victoria, F.J. Pérez-Victoria, A. Parodi-Talice, I.A. Jimenez, A.G. Ravelo, S. Castanys, F. Gamarro, Alkyl-lysophospholipid resistance in multidrug-resistant *Leishmania tropica* and chemosensitization by a novel P-glycoprotein-like transporter modulator, Antimicrob. Agents Chemother. 45 (2001) 2468–2474.
[55] F.J. Pérez-Victoria, M.P. Sánchez-Cañete, S. Castanys, F. Gamarro, Phospholipid translocation and miltefosine potency require both *L. donovani* miltefosine transporter and the new protein LdRos3 in *Leishmania* parasites, J. Biol. Chem. 281 (2006) 23766–23775.
[56] S.P. Ravaud, M.-A. Do Cao, M. Jidenko, C. Ebel, M. Le Maire, J.-M. Jault, A. Di Pietro, R. Haser, N. Aghajari, The ABC transporter BmrA from *Bacillus subtilis* is a functional dimer when in a detergent-solubilized state, Biochem. J. 395 (2006) 345–353.
[57] J.-L. Rigaud, D. Levy, D. Nejat, Reconstitution of membrane proteins into liposomes, Methods in Enzymology, Vol. 372, Academic Press, 2003, pp. 65–86.
[58] J.L. Rigaud, D. Levy, G. Mosser, O. Lambert, Detergent removal by non-polar polystyrene beads, Eur. Biophys. J. 27 (1998) 305–319.
[59] J. Chopineau, S. Lesieur, M. Ollivon, Vesicle formation by enzymic processes, J. Am. Chem. Soc. 116 (1994) 11582–11583.
[60] B. Carion-Taravella, S. Lesieur, M. Ollivon, J. Chopineau, Enzyme-mediated formation of vesicles from DPPC–dodecyl maltoside mixed micelles, J. Am. Chem. Soc. 120 (1998) 10588–10595.
[61] E. Katchalski-Katzir, D.M. Kraemer, Eupergit® C, a carrier for immobilization of enzymes of industrial potential, J. Mol. Catal. B Enzym. 10 (2000) 157–176.
[62] O. Lambert, D. Levy, J.-L. Ranck, G. Leblanc, J.-L. Rigaud, A new "Gel-like" phase in dodecyl maltoside–lipid mixtures: implications in solubilization and reconstitution studies, Biophys. J. 74 (1998) 918–930.
[63] C.A. Doige, X. Yu, F.J. Sharom, The effects of lipids and detergents on ATPase-active P-glycoprotein, Biochim. Biophys. Acta BBA Biomembr. 1146 (1993) 65–72.
[64] Y. Romsicki, F.J. Sharom, The ATPase and ATP-binding functions of P-glycoprotein, Eur. J. Biochem. 256 (1998) 170–178.
[65] O. Dalmas, M.-A. Do Cao, M.R. Lugo, F.J. Sharom, A. Di Pietro, J.-M. Jault, Time-resolved fluorescence resonance energy transfer shows that the bacterial multidrug ABC half-transporter BmrA functions as a homodimer, Biochemistry 44 (2005) 4312–4321.